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Cigarette Smoke and Nicotine-Induced Remodeling of Actin Cytoskeleton and Extracellular Matrix by Vascular Smooth Muscle Cells

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Cigarette smoking is a significant risk factor for atherosclerosis, which involves the invasion of vascular smooth muscle cells (VSMCs) from the media to intima. Many invasive cells remodel the actin cytoskeleton to form podosomes, which regulate metalloproteinase (MMP) release for extracellular matrix (ECM) degradation. We tested the hypothesis that cigarette smoke extract (CSE) modulates the structure and function of podosomes in VSMCs. We found that**, i**n response to PKC activation by phorbol dibutyrate (PDBu), untreated A7r5 VSMCs formed podosomes, whereas CSE, nicotine, and carbachol-treated cells formed actin-rich rings. The nicotinic acetylcholine receptor (nAChR) antagonist α-bungarotoxin abolished the effects of nicotine and carbachol on actin-rich ring formation. Immunofluorescence labeling experiments localized MMP-2 and nAChRs at the actin-rich rings. Nicotine-induced actin-rich ring formation required 6-12 hr exposure, and was sensitive to the protein synthesis inhibitor cycloheximide. Conditioned media collected from cell culture of nicotine-treated cells induced podosome remodeling in untreated cells. When cells were cultured on DQ-gelatin, untreated cells exhibited a fibrillar network of fluorescence from DQ-gelatin degradation, which, upon PDBu stimulation, reorganized into peripheral dots and migrated towards the perinuclear region. In contrast, nicotine-treated cells, upon PDBu stimulation, reorganized the fluorescence into perinuclear fibrils, which dispersed into small dots and disappeared rapidly over time. Results from this study suggest that nicotine, by activating nAChRs and inducing phenotypic modulation, may prime VSMCs to become hyperresponsive to PKC activation with an enhanced ability to remodel the actin cytoskeleton and release MMP for invasion of the ECM.
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Bupropion Binds in the Middle (M2-9) of the *Torpedo* Nicotinic Acetylcholine Receptor Ion Channel

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Bupropion is clinically prescribed for the treatment of depression (Wellbutrin) and for smoking cessation (Zyban). While there is consensus that the primary mechanism of action involves increased synaptic concentrations of dopamine and norepinephrine via inhibition of the respective reuptake transporters, DAT and NET, there is growing evidence that some of the therapeutic benefits of bupropion may result from non-competitive antagonism of neuronal nicotinic acetylcholine receptors (nAChRs), in particular α4β2 and α3β4 subtypes. To aid in elucidating the mechanism of bupropion action and the development of new therapeutic agents for smoking cessation and depression, our goal is to determine the specific molecular interactions between bupropion and several nAChR subtypes (*Torpedo*, α4β2, α3β4). We first established that bupropion dose-dependently and reversibly inhibits ACh-induced currents in *Xenopus laevis* oocytes injected with affinity-purified and lipid-reconstituted *Torpedo* nAChRs (IC_{50} 0.34 μM). Using a photoreactive analog of bupropion ([^{125}I]-SADU-3-72), we next established that bupropion inhibits the binding of [^{125}I]-SADU-3-72 to the *Torpedo* nAChR (IC_{50} closed state, 3.6 μM; desensitized state, 1.2 μM) and that binding to the closed nAChR is fully inhibited by tetracaine (IC_{50} 0.42 μM) consistent with a bupropion/[^{125}I]-SADU-3-72 binding site within the nAChR channel. [^{125}I]-SADU-3-72 photolabeled *Torpedo* nAChR subunits in an agonist-sensitive and bupropion-inhibitable (specific) manner. Finally for the closed nAChR, we identified sites of specific [^{125}I]-SADU-3-72 labeling within δM2 (δLeu265/δM2-9) and βM2 (βLeu257/βM2-9). In the desensitized state, TCP-inhibitable (specific) labeling was identified within δM2 (δLeu265, δM2-9 with minor labeling of δSer258, δM2-2). Our results establish that bupropion binds in the middle (M2-9) of the *T*orpedo nAChR channel, with a slightly broader binding locus in the desensitized channel. Currently, studies are underway to identify the site(s) of [^{125}I]-SADU-3-72 labeling in affinity-purified α4β2 and α3β4 nAChRs.

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Injection of Affinity-Purified Ligand-Gated Ion Channels into *Xenopus Laevis* Oocytes for Functional Studies using Electrophysiology

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Heterologous expression after mRNA/cDNA injection into *Xenopus* oocytes is a well established system for studying the structure and function of a variety of membrane proteins including ligand-gated ion channels (LGIC). In the early 1990's, the Miledi group [1] introduced a rather unique variant of this system by injecting pre-assembled nicotinic acetylcholine receptors (nAChR) into oocytes and measuring function using electrophysiological recordings. Native or affinity-purified *Torpedo* nAChR-membranes were injected into *Xenopus* oocytes and after incubation to allow receptor-containing membranes to fuse with the plasma membrane, functional receptor channels were detected using two-electrode voltage clamp recordings. Our goal is to utilize this system to assess the functionality and pharmacology of affinity-purified and lipid-reconstituted LGICs. We first determined that the optimal incubation period for the appearance of maximal functional channels following injection of native or affinity-purified and lipid-reconstituted *Torpedo* nAChRs was 48 hr. We next established that injection of affinity-purified *Torpedo* nAChRs reconstituted in lipid vesicles comprised of only phosphatidylcholine (DOPC) had minimal functionality (300 μM i.e. $>EC_{100}$ ACh-induced currents at −60 mV; ~ 13-23 nA). In contrast, affinity-purified *Torpedo* nAChRs reconstituted in vesicles comprised of DOPC/DOPA/CH (3:1:1) were fully functional (300 μM ACh at −60 mV; ~ 1-3 μA) and the EC_{50} for ACh (~ 50 μM) was comparable to that obtained for *Torpedo* nAChR mRNA injected oocytes. These results indicate that CH and anionic lipids (PA) are not only required for receptor functionality [2] but are protective of receptors during detergent solubilization and affinity-purification. Currently, studies are underway to examine the functionality of several affinity-purified and lipid-reconstituted LGICs, including α4β2 and α3β4 nAChRs, and 5-HT_{3A} receptors.

[1] Marsal et al (1995) *PNAS* 92, 5224-5228.
[2] Hamouda et al (2006) *Biochemistry* 45, 4327-4337.

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Sources of Energy for Gating by Agonists in Acetylcholine Receptor-Channels

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The neuromuscular acetylcholine receptor (AChR) is an allosteric protein that switches between closed- and open-channel conformations. The probability of being open increases when acetylcholine (ACh) molecules occupy the two transmitter binding sites (TBS). Based on the MWC model and experimental measures of the di- and unliganded gating equilibrium constants (E_2 and E_0), we estimate the energy that an agonist provides to power gating is $=0.59\ln(\sqrt{(E_2/E_0)})$. In wt adult neuromuscular AChRs, each ACh provides ~-5.1 kcal/mol. The question we asked is: which TBS residues provide this energy? Using single-channel electrophysiology, we examined four aromatic residues in the α subunit, Y93, W149, Y190, and Y198. Mutation to Phe caused small (<1 kcal/mol) reductions in energy at positions 93, 149, and 198, but a large reduction effect at 190. The removal of aromatic ring by Ala mutation caused large (≥2 kcal/mol) reductions at positions 149, 190, and 198, but not 93. These results suggest that 149 and 198 interact with ACh mainly through the aromatic ring, not H-bonding. Mutations of 190 to F, W, and A had the largest reductions (2.2-4.5 kcal/mol), so this residue provides most of the energy. In contrast, 93 is much weaker as mutations to F, W, H, A, C, S, and G reduced the energy ≤1.1 kcal/mol. Overall, the order of energy loss was 190>>149>198>93. The sum of the Ala mutant energy reductions for the four positions was >5.1 kcal/mol, suggesting that these side chains interact energetically. We created double mutant F-F constructs (n=6) and observed significant coupling (>1.5 kcal/mol) between 190-93, 190-149, and 190-198, but little coupling between the other pairs. Thus, 190 not only provides most of the energy for gating, but is also coupled with the surrounding aromatic TBS residues.
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Effects of Lipid-Analog Detergent Solubilization on the Functionality and Lipidic Cubic Phase Mobility of the Torpedo Californica Nicotinic Acetylcholine Receptor

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Over the past three decades, the Torpedo californica nicotinic acetylcholine receptor (nAChR) has been one of the most extensively studied membrane protein systems. However, the effects of detergent solubilization on nAChR resistance to degradation and function are poorly understood. The use of lipid-analog detergents for nAChR solubilization has been shown to preserve receptor integrity and functionality. The present study used lipid-analog detergents from phospholipid-analog and cholesterol-analog detergent families for solubilization and affinity purification of the receptor and probed nAChR ion